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Dopamine D2 receptors bidirectionally regulate striatal enkephalin expression: Implications for cocaine reward

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SUMMARY

Low dopamine D2 receptor (D2R) availability in the striatum can predispose for cocaine abuse; though how low striatal D2Rs facilitate cocaine reward is unclear. Overexpression of D2Rs in striatal neurons or activation of D2Rs by acute cocaine suppresses striatal *Penk* mRNA. Conversely, low D2Rs in D2-striatal neurons increases striatal *Penk* mRNA and enkephalin peptide tone, an endogenous mu-opioid agonist. In brain slices, met-enkephalin and inhibition

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AUTHOR CONTRIBUTIONS

Conceptualization and Methodology: L.K.D. and V.A.A. Investigation and Analysis: L.K.D., I.B.C., K.Z.D., R.L., J.H.S., A.M., C.K., E.H.S., M.B.B., and A.R.K. Writing – Original Draft: L.K.D. and V.A.A. Review and Editing: L.K.D., V.A.A., C.K., E.H.S., M.E.B. Supervision: L.K.D., V.A.A., M.E.B. Resources: L.K.D., V.A.A., C.K., and E.H.S.

SUPPLEMENTAL INFORMATION

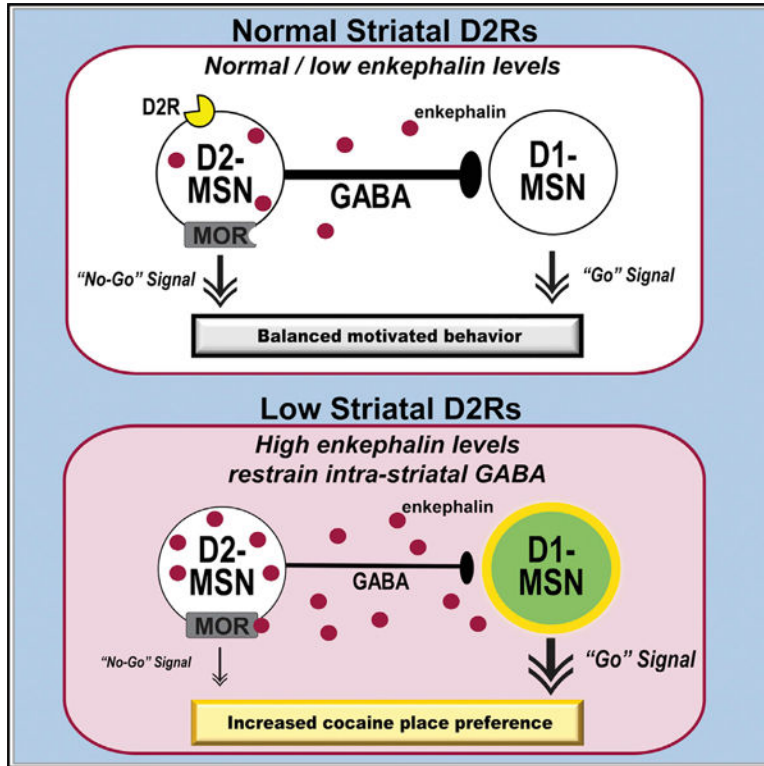
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DECLARATION OF INTERESTS

The authors declare no competing interests.

of enkephalin catabolism suppresses intra-striatal GABA transmission. Pairing cocaine with intra-accumbens met-enkephalin during place conditioning facilitates acquisition of preference, while mu-opioid receptor antagonist blocks preference in wild-type mice. We propose that heightened striatal enkephalin potentiates cocaine reward by suppressing intra-striatal GABA to enhance striatal output. Surprisingly, a mu-opioid receptor antagonist does not block cocaine preference in mice with low striatal D2Rs, implicating other opioid receptors. The bidirectional regulation of enkephalin by D2R activity and cocaine offers insights into mechanisms underlying the vulnerability for cocaine abuse.

Graphical abstract



In brief

Low striatal D2 receptor levels are associated with cocaine abuse. Dai et al. bidirectionally alter striatal D2 receptor levels to probe the downstream mechanisms underlying this abuse liability. They provide evidence that enhanced enkephalin tone resulting from low D2 receptors is associated with suppressed intra-striatal GABA and potentiated cocaine reward.

INTRODUCTION

Cocaine addiction is a prevalent substance use disorder (SUD) that not only incurs substantial costs for the user’s physical health, but also for interpersonal relationships and society at large. With no clinically approved efficacious treatments available, identifying the neurobiological underpinnings that predispose individuals to develop a cocaine SUD is a

critical step toward advancing preventive medicine. One well-reported vulnerability factor for cocaine addiction is low levels of the dopamine D2 receptor (D2R) within the striatum, a key region in the brain's mesolimbic reward pathway (Dalley et al., 2007; Michaelides et al., 2012; Volkow et al., 2006). We previously generated a mouse model of low striatal D2Rs and found that selective deletion of the *Drd2* gene from indirect pathway striatal medium spiny neurons (MSN-*Drd2*KO) enhances cocaine place preference and facilitates cocaine locomotor sensitization (Dobbs et al., 2016, 2019). While this cell-type specific knockout animal has become a useful model for studying addiction vulnerability, the exact downstream mechanisms by which low striatal D2Rs predispose individuals to cocaine abuse still remain relatively unknown. One potential mechanism is through D2R-mediated regulation of enkephalins, which are endogenous opioid peptides implicated in cocaine reward and locomotor sensitization.

Enkephalins are mainly derived from the preproenkephalin gene (*Penk*), which generates both met- and leu-enkephalin, and to a lesser extent from the prodynorphin gene (*Pdyn*), which produces leu-enkephalin after cleavage of dynorphin (Akil et al., 1984; Day et al., 1998; Gianoulakis, 2005; Weisinger, 1995). Enkephalins have high affinity for the μ - and δ -opioid receptors (MOR and DOR, respectively), and are expressed throughout the brain, with the striatum being one of the highest expressing regions. In the striatum, D2R-containing MSNs express *Penk* and are the primary source of enkephalin tone in this region (Gerfen and Surmeier, 2011; Lemos et al., 2016). Multiple reports indicate that the activity level of D2Rs regulates the expression levels of enkephalin in the striatum. Dopamine depletion using 6-OHDA leads to a marked enhancement in striatal *Penk* mRNA expression, which can be reversed by chronic administration of the D2-like receptor agonist, quinpirole (Gerfen et al., 1990). This suggests that low activity of D2Rs upregulates *Penk* mRNA in the striatum, while activation of D2Rs downregulates it. Other experiments using global, constitutive *Drd2* knockout mice or the D2-like receptor antagonist, haloperidol, provide further independent evidence that loss of D2R signaling causes upregulation of striatal enkephalin expression (Baik et al., 1995; Romano et al., 1987). Since these previous studies manipulated D2R activity in a global fashion, it remains unknown which type of D2R-expressing cells are responsible for regulating striatal enkephalin expression. This is an important consideration since D2Rs are expressed not only in MSNs, but also in cholinergic interneurons and on axonal projections from midbrain dopamine neurons and cortical and hippocampal glutamatergic neurons (Bamford et al., 2004; Bello et al., 2011; Higley and Sabatini, 2010; Maurice et al., 2004; Sesack et al., 1994).

Cocaine, administered repeatedly at moderate doses or at a "binge-like" high dose, has also been reported to increase *Penk* mRNA levels in the striatum (Crespo et al., 2001; Mantsch et al., 2004; Mathieu-Kia and Besson, 1998; Mongi-Bragato et al., 2016), but see two reports showing the opposite (Branch et al., 1992; Daunais et al., 1997). It is speculated that a history of cocaine taking induces an enhancement in striatal enkephalin tone that is responsible for opioid receptor-dependent long-term depression (LTD) of the striatopallidal pathway (Creed et al., 2016; Kupchik et al., 2014). Modulation of this striatopallidal pathway activity has been shown to provoke cocaine seeking (Heinsbroek et al., 2017); however, whether heightened striatal enkephalin actually drives cocaine seeking remains to be determined.

The activity of MORs is important in signaling the motivational and affective aspects of rewards, which, through careful dissection of regional differences, have been mapped as hedonic *hotspots* and *coldspots* in the ventral aspect of the striatum, also known as the nucleus accumbens (NAc) (Castro and Berridge, 2014; Castro and Bruchas, 2019; Peciña and Berridge, 2005). Considering the high expression level of enkephalin in the NAc and the rest of the striatum, and high affinity of this endogenous peptide for MORs, it is reasonable to hypothesize that enkephalin contributes to MOR-mediated reward. In support of this hypothesis, a previous study showed that enkephalin is reinforcing in its own right, as rats will self-administer met-enkephalin into the NAc, as well as selective MOR or DOR agonists (Goeders et al., 1984; Simmons and Self, 2009).

Other studies provide further evidence that endogenous opioids, likely enkephalin, and opioid receptors in the NAc, play an important role in mediating cocaine reward and reinforcement. For example, microinjection of the MOR antagonist, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP), into the NAc suppressed cocaine-induced reinstatement of drug seeking (Simmons and Self, 2009) and cocaine place preference (Soderman and Unterwald, 2008). While this CTAP effect could have been mediated by β -endorphins, which also have high affinity for MORs, studies using genetic manipulations point to enkephalin as being an important player in cocaine reinforcement. Global deletion of *Penk* or the DOR gene (*Oprd1*) suppresses acquisition of cocaine self-administration and the breakpoint for cocaine, while knockout of the DOR or MOR gene (*Oprm1*) suppresses cue-induced reinstatement of cocaine seeking (Gutiérrez-Cuesta et al., 2014). However, since these effects are the result of global *Penk*, *Oprd1*, *Oprm1* knockout, it is unclear whether enkephalin signaling in the striatum, per se, is sufficient to potentiate cocaine reward. Further, understanding how D2Rs modulate enkephalin expression is likely to unveil the mechanism by which low striatal D2Rs contribute to the addiction vulnerable phenotype and also provide new targets for preventive therapeutics that address vulnerability.

Here, we tested the hypothesis that low levels of D2R activation in the striatum triggers increased enkephalin expression, release, and functional tone, which potentiates cocaine conditioned place preference and eventually contributes to increased vulnerability for abuse. We manipulated the activity levels of striatal D2Rs using viral and genetic techniques and measured enkephalin expression levels and functional tone in the striatum using quantitative PCR and *ex vivo* electrophysiology, respectively. In a separate set of experiments, we tested whether heightened striatal enkephalin tone facilitates cocaine reward in a place conditioning procedure.

RESULTS

Dopamine D2 receptor expression in striatal medium spiny neurons regulates preproenkephalin expression

We bidirectionally manipulated D2R expression in the striatum (overexpression and knockdown) using two approaches that allow for temporal and cell-type specificity. First, we used an inducible tet-Off system to transiently overexpress the human *Drd2* gene in adult mice (Kellendonk et al., 2006). This approach was previously shown to direct *Drd2* expression to striatal MSNs and increase striatal D2R binding by 15% (Kellendonk et al.,

2006). Even though this approach is not designed to have cell-type specificity of transgene expression, overexpression of *Drd2* was enriched in *Penk*-positive D2-MSNs (40%) versus D1R-expressing MSNs (25%) (Labouesse et al., 2018). Here, we measured levels of *Penk* RNA in the striatum of *Drd2* overexpressing (D2R-OE) mice and their control littermates.

Expression of the *Drd2* transgene in D2R-OE mice was switched off by feeding with doxycycline-supplemented chow for 2 weeks (ON Dox) (Kellendonk et al., 2006). During ON Dox we found no difference in levels of *Penk* RNA between D2R-OE and control mice (Figures 1A and 1B). In absence of doxycycline treatment when mice were fed normal chow (OFF Dox), the relative levels of striatal *Penk* RNA were reduced by 50% in D2R-OE mice compared with controls (0.50 ± 0.06 versus 1.02 ± 0.09 ; $t_7 = 4.3$, $p = 0.0036$; Figure 1B). In addition, gene chip microarray analysis showed that D2R-OE mice have an 18% decrease in cRNA for *Penk* in the striatum compared with controls (7626 ± 110 versus 9266 ± 145 , respectively; $t_8 = 9.0$, $p < 0.0001$; Figure 1C). Together, this indicates that overexpression of D2Rs in striatal MSNs results in a reliable reduction of *Penk* gene transcription (OFF Dox: Spearman's $r = 0.82$, $p < 0.01$; Figure 1D).

Conversely, reducing striatal D2R levels with targeted deletion of the *Drd2* gene from D2-MSNs increased *Penk* expression. Striatal samples from mice with deletion of both *Drd2* alleles in D2-MSNs have >80% reduction of *Drd2* RNA (Figure 1E; Dobbs et al., 2016; Lemos et al., 2016) and a $56\% \pm 5.3\%$ increase in *Penk* RNA relative to littermate controls, which have unaltered D2R levels ($t_5 = 5.19$, $p < 0.01$; Figure 1F). Next, we investigated whether there were adaptive alterations in MORs, DORs, or dynorphin (*Pdyn*), an opioid peptide expressed by D1R-expressing MSNs. We found no difference in expression levels of *Pdyn* ($t_4 = 0.08$, $p > 0.05$), the MOR gene *Oprm1* ($t_6 = 1.2$, $p > 0.05$), or the DOR gene *Oprd1* ($t_5 = 0.17$, $p > 0.05$) in the striatum of MSN-*Drd2*KO mice compared with littermate controls.

Cocaine bidirectionally alters striatal *Penk* mRNA via D2Rs in MSNs depending on length of treatment

Long-term cocaine treatment ($15 \text{ mg/kg} \times 10 \text{ days}$) is reported to increase *Penk* mRNA expression in the striatum (Crespo et al., 2001; Mantsch et al., 2004; Mathieu-Kia and Besson, 1998; Mongi-Bragato et al., 2016). Evidence suggests this effect is through long-term cocaine treatment downregulating D2R activity (Baik et al., 1995; Moore et al., 1998; Nader et al., 2002, 2006; Romano et al., 1987; Steiner and Gerfen, 1998) Moore et al., 1998a,b. Given our data showing the connection between striatal *Drd2* and *Penk* expression, we hypothesized the length of cocaine treatment would similarly bidirectionally regulate striatal *Penk* expression by targeting D2Rs in MSNs.

We replicated reports in the literature and found that long-term, non-contingent cocaine administration ($20 \text{ mg/kg} \times 10 \text{ days}$) followed by 14 days of withdrawal significantly increased *Penk* expression in the striatum of C57Bl/6J mice compared with saline-treated controls ($t_{10} = 2.24$, $p < 0.05$; Figure 1G). We observed a similar increase in *Penk* expression in wild-type mice following 14-day withdrawal from 10 days of cocaine self-administration ($17.4 \pm 2.8 \text{ mg/kg}$ cocaine per day) compared with those that self-administered saline ($t_{10} = 2.62$, $p < 0.05$; Figure 1H). Conversely, wild-type mice exposed to a short-term,

non-contingent cocaine regimen (15 mg/kg \times 5 days) followed by 14 days of withdrawal showed significantly less striatal *Penk* expression compared with saline-treated controls ($t_9 = 2.85$, $p < 0.05$, Figure 1I). Thus, long-term and short-term cocaine exposure followed by withdrawal bidirectionally regulates striatal *Penk* expression in a similar manner to striatal D2R activity. We next tested whether D2Rs specifically in MSNs were mediating cocaine's effect by replicating the short-term cocaine experiment in MSN-*Drd2*KO mice. As expected, short-term cocaine followed by withdrawal failed to decrease striatal *Penk* expression in MSN-*Drd2*KO mice as it did in wild type. MSN-*Drd2*KO mice showed no change in *Penk* expression after short-term cocaine treatment compared with saline-treated MSN-*Drd2*KO mice ($t_9 = 1.25$, $p = 0.2$). Together, these data indicate that activity of MSN D2Rs bidirectionally regulate striatal *Penk* expression, and that cocaine impinges upon these D2Rs to similarly regulate *Penk* expression.

Met-enkephalin and MOR agonist suppress intra-striatal GABA transmission

We next assessed the physiological response to met-enkephalin in the nucleus accumbens (NAc) by testing how it affects collateral GABA transmission using *ex vivo* electrophysiological recordings. Since *Gi*-coupled dopamine receptors potently suppress collateral GABA transmission in the NAc (Dobbs et al., 2016; Gallo et al., 2018), we hypothesized that met-enkephalin would have a similar effect via activation of *Gi*-coupled opioid receptors. Met-enkephalin has high affinity for the DOR and MOR, and both are expressed in MSNs (Gracy et al., 1997; Mansour et al., 1994; Svingos et al., 1996, 1997).

We expressed channelrhodopsin-2 (ChR2) in D2-MSNs using Cre-dependent vectors injected in the NAc of MSN-*Drd2*KO mice and *Adora2a*Cre controls. This enabled optogenetic stimulation of collateral axons from D2-MSNs while recording from neighboring ChR2-negative MSNs to measure GABA-A receptor-mediated inhibitory postsynaptic currents (IPSCs).

We first tested the selective MOR agonist DAMGO (1 μ M) in *Adora2a*-Cre controls (Figure 2A). DAMGO robustly suppressed the amplitude of optogenetic-evoked IPSCs (oIPSCs) to $35\% \pm 4\%$ of baseline (1W-RMANOVA, $F_{1,04, 4.18} = 38.59$, $p < 0.01$; baseline versus DAMGO: $t_4 = 16.43$, $p < 0.001$). This was reversed by the non-selective opioid receptor antagonist naloxone (5 μ M; baseline versus naloxone: $t_4 = 1.56$, $p = 0.4$). Similarly, bath application of met-enkephalin (1 μ M), in the presence of peptidase inhibitors to slow met-enkephalin degradation (2 μ M thiorphan, 10 μ M bestatin), robustly inhibited oIPSC amplitude relative to peptidase inhibitor baseline (2W-Mixed Model, main effect of drug: $F_{2,30} = 83.4$, $p < 0.0001$) (Figures 2B and 2C). Maximal inhibition was similar between genotypes (MSN-*Drd2*KO: $42\% \pm 8\%$ of baseline; *Adora2a*Cre: $33\% \pm 5\%$ of baseline; $t_{49} = 1.2$, $p = 0.6$), suggesting no apparent alterations in the function of MORs or DORs in MSN-*Drd2*KO mice. Naloxone (5 μ M) reversed inhibition by met-enkephalin similarly between MSN-*Drd2*KO and *Adora2a*Cre controls ($t_{49} = 1.7$, $p = 0.2$).

We next measured whether met-enkephalin also inhibits spontaneously generated IPSCs (sIPSCs) in this same preparation. As we observed previously, MSN-*Drd2*KOs have a higher striatal GABA tone than controls (Lemos et al., 2016), which was reflected by a greater baseline sIPSC amplitude (K-S test, $D = 0.64$, $p < 0.05$; Figures 2D–2F). Met-enkephalin

attenuated the overall mean sIPSC amplitude in MSN-*Drd2*KOs, but not controls, when compared with peptidase inhibitor baseline (data not shown); however, we suspected that averaging the amplitude data might mask an effect in the controls. Thus, we built frequency histograms of sIPSC amplitude size and compared the amplitudes between drug washes and genotypes. Met-enkephalin suppressed sIPSCs in controls relative to baseline (Wilcoxon signed rank test: $p < 0.05$; Figure 2E), an effect likely masked in the analysis of the overall mean. Met-enkephalin also suppressed sIPSCs in MSN-*Drd2*KOs compared with baseline (2W-RMANOVA, amplitude \times drug: $F_{20,140} = 3.42$, $p < 0.0001$; Figure 2F). Further, comparison of the sIPSC distributions using a K-S test revealed that met-enkephalin suppressed sIPSCs significantly more for MSN-*Drd2*KOs than controls ($D = 0.64$, $p < 0.05$). This effect is supported by the wider range of met-enkephalin suppression at both small (10 pA: $t_{140} = 2.3$, $p < 0.05$) and large (100 pA: $t_{140} = 2.2$, $p = 0.05$) amplitude responses for the MSN-*Drd2*KOs.

Subsequent application of naloxone (5 μ M) resulted in an overshoot of sIPSC amplitude over baseline, thus revealing the presence of a baseline opioid peptide tone. In MSN-*Drd2*KOs, naloxone enhanced GABA transmission for a wide range of amplitudes (10 pA–60 pA; post hoc t tests: t 's = 2.1–5.8, p 's < 0.01–0.0001). However, while naloxone enhanced sIPSCs in controls (Wilcoxon signed rank test: $p < 0.05$), comparison of the distributions revealed that naloxone enhanced GABA transmission significantly more in MSN-*Drd2*KOs than controls (K-S test: $D = 0.64$, $p < 0.05$). The larger naloxone effect in the MSN-*Drd2*KO mice was the first indication of an enhanced functional tone of enkephalin in mice with low striatal D2Rs.

Met-enkephalin did not suppress the sIPSC frequency (2W-RMANOVA, drug: $F_{2,20} = 13.18$, $p < 0.001$; $t_{20} = 1.5$, $p = 0.3$). However, similar to the effect on amplitude, naloxone significantly increased sIPSC frequency relative to baseline for both genotypes ($t_{20} = 3.5$, $p < 0.01$; Figure 2G). Together, these data indicate that exogenously applied met-enkephalin acts on opioid receptors to inhibit small amplitude synaptic GABA responses. Further, opioid receptor antagonist revealed the presence of an opioid peptide tone in the striatum that appears stronger in animals with low D2Rs.

Evidence of heightened endogenous tone of enkephalin in MSN-*Drd2*KO mice

The presence of a higher tone of striatal met-enkephalin is consistent with the elevated *Penk* mRNA expression observed in MSN-*Drd2*KOs. To address this further, we carried out two sets of experiments to test for evidence of this electrophysiologically and behaviorally. Electrophysiologically, we assessed the ability of a peptidase inhibitor cocktail to inhibit collateral GABA on its own. The peptidase inhibitors thiorphan and bestatin raise met-enkephalin extracellular levels in the striatum by inhibiting the enzymes that degrade met-enkephalin (Nam et al., 2019). This peptidase inhibitor cocktail was effective in suppressing glutamate transmission in the amygdala by enhancing enkephalin responses (Gregoriou et al., 2020). We therefore hypothesized peptidase inhibitors would be more effective at suppressing striatal GABA transmission in MSN-*Drd2*KO mice than controls. We found that application of peptidase inhibitors suppressed the amplitude of oIPSCs and the frequency and amplitude of sIPSCs recorded from NAc MSNs (Figures 3 and S1).

The suppression of oIPSC amplitude was only significant in MSN-*Drd2*KO mice ($76\% \pm 4\%$ from baseline versus $96\% \pm 5\%$ of baseline in controls; 2W-Mixed Model, drug \times genotype: $F_{1,35} = 11.75$, $p < 0.01$; MSN-*Drd2*KO versus *Adora2a*Cre: $t_{35} = 4.8$, $p < 0.0001$; Figures S1A–S1D). The suppression of overall sIPSC mean amplitude was also only significant in MSN-*Drd2*KOs and not in controls (data not shown); however, comparison of the distributions of sIPSC amplitude size showed that peptidase inhibitors had a similar effect on both controls and MSN-*Drd2*KOs (K-S test: $D = 0.55$, $p = 0.08$; Figures 3A and 3B). Peptidase inhibitors reduced sIPSC amplitude in controls (Wilcoxon signed rank test: $p < 0.001$) and MSN-*Drd2*KOs (2W-RMANOVA, drug: $F_{1,77} = 50.1$, $p < 0.0001$). Similarly, peptidase inhibitors reduced sIPSC frequency equally between genotypes (2W-RMANOVA, drug: $F_{1,10} = 6.24$, $p < 0.05$; Figure 3D). Pre-incubation with the opioid receptor antagonist naloxone ($5 \mu\text{M}$) blocked the effect of peptidase inhibitors to inhibit sIPSC frequency (baseline: 21 ± 6 , peptidase inhibitors: 32 ± 6 sIPSCs/15 s; $t_5 = 2.9$, $p < 0.05$). However, to our surprise, pre-incubation with naloxone did not block the inhibition of oIPSC amplitude, suggesting the possibility that another neuropeptide is involved. Receptor antagonists for Substance P and nociceptin, two peptides highly expressed in the striatum, also did not block the suppression of oIPSCs by peptidase inhibitors. Pre-incubation with the neurokinin1 antagonist Aprepitant ($1 \mu\text{M}$) and nociceptin antagonist J 113397 ($1 \mu\text{M}$) failed to block oIPSC suppression induced by peptidase inhibitors (one-sample t tests, baseline versus antagonists: t 's = 4.5–6.7, p 's < 0.01 ; Figure S1D). Taken together, the data indicate that while peptidase inhibitors suppressed GABA transmission in controls and MSN-*Drd2*KOs, the effect is more robust in MSN-*Drd2*KO mice. Further, it appears that peptidase inhibitors exert their effect by raising levels of opioid and non-opioid peptides.

We next probed for *in vivo* evidence of heightened enkephalin tone in mice with low D2Rs and sought to determine the consequences for motivated behavior. Endogenous met-enkephalin tone is important for maintaining a basal hedonic set-point. Administration of naloxone during place conditioning induces a place aversion in mice (Cunningham et al., 1995; Solecki et al., 2009). Evidence from *Penk* and *Oprm1* constitutive knockout mice suggests naloxone induces place aversion by blocking the action of enkephalin on MORs (Skoubis et al., 2001, 2005). We reasoned that if MSN-*Drd2*KO mice have higher endogenous enkephalin tone, they might be more sensitive to developing and expressing place aversion to a low dose of naloxone (Figure 4A). As we observed previously (Dobbs et al., 2016, 2019), MSN-*Drd2*KO mice showed less locomotor activity compared with littermate controls during saline and naloxone (1 mg/kg , intraperitoneally) conditioning trials (3W-RMANOVA, genotype: $F_{1,13} = 27.3$, $p < 0.001$; Figure 4B). We assessed naloxone place aversion by comparing the time spent on the grid floor between the conditioning subgroups (Grid + Nal versus Hole + Nal). In this approach, a place aversion is expressed as less time spent on the grid floor for the Grid + Nal group than the Hole + Nal group. We predicted MSN-*Drd2*KO mice would spend less time on the naloxone paired floor compared with controls. However, neither genotype showed evidence of naloxone place aversion during saline-primed preference tests (Tests 1 and 2: one-sample t test versus 50%: p 's > 0.6 ; Figure 4C). This was confirmed by additional analysis of the grid time data (Test 1: 2W-ANOVA, conditioning: $F_{1,9} = 0.57$, $p = 0.5$; Test 2: 2W-ANOVA, conditioning: $F_{1,11} = 0.39$, $p = 0.5$; Figure 4D). Though, when tested in the presence of naloxone,

MSN-*Drd2*KO mice spent significantly less percent time on the naloxone paired floor than controls ($t_{13} = 2.5$, $p < 0.05$). Analysis of time spent on the grid floor showed the same effect in MSN-*Drd2*KO mice (2W-ANOVA, genotype \times conditioning: $F_{1,11} = 5.49$, $p < 0.05$; MSN-*Drd2*KOs: $t_{11} = 2.3$, $p = 0.08$; Controls: $t_{11} = 1.0$, $p = 0.3$). Importantly, naloxone had no effect on locomotion, indicating the place aversion was not due to locomotion suppression (Figure S2).

Together, the electrophysiology and place preference data provide evidence for heightened met-enkephalin tone in the striatum of MSN-*Drd2*KO mice, and suggest this tone has consequences for motivated behavior. Further, these data suggest that met-enkephalin and peptidase inhibitors suppress collateral GABA transmission through activation of MORs.

Nucleus accumbens opioid receptors facilitate cocaine reward

MSN-*Drd2*KO mice acquire cocaine place preference faster than littermate controls (Dobbs et al., 2016). We wondered whether the heightened enkephalin tone and signaling through MORs could be contributing to the enhanced cocaine place preference (CPP), since blockade of MORs in the NAc of rats attenuates cocaine place preference (Soderman and Unterwald, 2008). To this end, we tested whether MOR signaling in the NAc facilitates expression of CPP in wild-type mice and whether it underlies the enhanced acquisition of cocaine preference in MSN-*Drd2*KO mice.

Control mice (*Drd2^{loxP/loxP}*) received cocaine place conditioning trials over 8 days, with preference measured before and after conditioning (Pre-test and Post-test; Figure 5A). A second Post-test was performed following intra-NAc shell CTAP administration to determine the contribution of MORs to the expression of cocaine CPP (CTAP test). Mice acquired cocaine CPP as indicated by a significant increase in the percent time spent on the cocaine-paired floor at Post-test compared with the Pre-test (1W-Mixed Model: $F_{1,61,16.91} = 4.3$, $p < 0.05$; Pre-test versus Post-test: $t_{13} = 2.98$, $p < 0.05$; Figure 5B). Blockade of MORs in the NAc shell (CTAP 300 ng/side) reduced the percent time spent on the cocaine paired floor (Post-test: $60\% \pm 4\%$, CTAP test: $53\% \pm 6\%$). In addition, the percent time spent on the cocaine floor during the CTAP test was not significantly different from at Pre-test ($t_8 = 1.04$, $p = 0.7$), suggesting that CTAP blocked the expression of CPP. This effect is further supported by analysis of the grid time data (2W-Mixed Model, conditioning \times test: $F_{2,31} = 4.49$, $p < 0.05$; Pretest: $t_{31} = 0.82$, $p = 0.8$; Post-test: $t_{31} = 3.42$, $p < 0.01$; CTAP test: $t_{31} = 0.99$, $p = 0.7$; Figure 5C). Importantly, locomotor activity during the CTAP test was not higher than the Post-test, which is an effect that can mask a place preference, and instead was slightly lower (Mixed Model: $F_{1,9, 19.9} = 3.64$, $p < 0.05$; $t_8 = 3.82$, $p < 0.05$; Figure S3). Together, these data indicate that activation of MORs in the NAc shell are necessary for the full expression of CPP and extend findings from rats to a mouse model.

Next, we tested whether blocking MORs during cocaine conditioning would attenuate acquisition of cocaine CPP in MSN-*Drd2*KO mice (Figure 5D). Intra-NAc CTAP or vehicle was administered immediately prior to cocaine conditioning trials. After only two cocaine and two saline conditioning trials, both the intra-NAc vehicle and intra-NAc CTAP groups showed significantly greater percent time on the cocaine-paired floor compared with Pre-test (2W-RMANOVA, test: $F_{2,24} = 7.02$, $p < 0.001$; Pre-test versus Saline Post-test: $t_{24} = 3.2$, p

< 0.01; Figure 5E). This was confirmed by analysis of the time spent on the grid floor (2W-ANOVA, conditioning: $F_{1,10} = 35.0$, $p < 0.001$; Figure 5F). Thus, blockade of MORs in the NAc did not attenuate acquisition of cocaine CPP in MSN-*Drd2*KO mice. However, since these MSN-*Drd2*KO mice have enhanced enkephalin tone throughout the entire striatum, it is possible that just blocking MORs in the NAc is not sufficient to attenuate CPP acquisition. Further, enkephalin also has high affinity for DORs, and activation of these receptors may contribute to the enhanced CPP acquisition. To address these possibilities, we tested these mice following a systemic injection of the non-selective opioid receptor antagonist naloxone (10 mg/kg). Surprisingly, naloxone did not block expression of cocaine CPP in either group, as indicated by greater percent time spent on the cocaine floor on the naloxone test versus the Pre-test ($t_{24} = 3.3$, $p < 0.01$), as well as by analysis of time spent on the grid floor for each treatment group (2W-ANOVA, conditioning: $F_{1,10} = 9.14$, $p < 0.05$). These data indicate that while MOR signaling sustains CPP in wild-type control mice, blocking MOR signaling is not sufficient to attenuate the faster acquisition of cocaine preference previously reported in MSN-*Drd2*KO mice.

Heightened enkephalin in the nucleus accumbens facilitates cocaine reward

Evidence from global enkephalin knockout mice and pharmacological manipulation of MORs and DORs suggest that striatal enkephalin contributes to the rewarding and locomotor sensitizing effects of cocaine (Gutiérrez-Cuesta et al., 2014; Mongi-Bragato et al., 2016). Although blockade of MORs does not attenuate CPP acquisition in MSN-*Drd2*KOs, these mice also have enhanced D1R signaling, which could mask the potentiating effects of enkephalin or even act synergistically with enhanced enkephalin to potentiate cocaine CPP. To test whether heightened striatal enkephalin alone is sufficient to potentiate the conditioned rewarding effects of cocaine, we paired cocaine with intra-NAc shell administration of either enkephalin or vehicle in wild-type *Drd2^{fl/fl}* mice during conditioning (Figure 6A). Intra-NAc shell enkephalin did not affect locomotion during conditioning (3W-Mixed Model, no enkephalin main effect or interaction), but cocaine increased locomotion compared with saline (trial \times cocaine: $F_{2,56, 64.87} = 4.74$, $p < 0.01$; post hoc t tests: $t = 4.3$ – 11.3 , $p < 0.001$; Figure S4).

Neither the vehicle nor enkephalin paired groups showed a significant pre-conditioning preference (vehicle: 46 ± 3 , enkephalin: $51\% \pm 4\%$ time on cocaine-paired floor; one-sample t tests, p 's > 0.3 ; unpaired t test vehicle versus enkephalin: $t_{27} = 0.87$, $p = 0.4$; Figure S4). After 1 week of conditioning, both groups spent greater than 50% time on the cocaine-paired floor (2W-ANOVA, test: $F_{2,83} = 10.7$, $p < 0.0001$; Pre-test versus Test 1: $t_{83} = 4.13$, $p < 0.001$). However, when compared with the NAc vehicle group, the NAc enkephalin group showed a strong trend to spend more percent time on the cocaine floor ($t_{28} = 1.94$, $p = 0.06$; Figure 6B). This enhanced acquisition was also observed when analyzing the time spent on the grid floor. While both the NAc vehicle and NAc enkephalin groups showed preference for the cocaine-paired floor (conditioning: $F_{1,26} = 40.57$, $p < 0.0001$), there was a strong trend for greater preference in the NAc enkephalin group (conditioning \times enkephalin: $F_{1,26} = 3.67$, $p = 0.06$; vehicle: $t_{26} = 3.03$, $p < 0.05$, enkephalin: $t_{26} = 6.1$, $p < 0.0001$). The potentiated cocaine preference in the enkephalin group, though, did not persist after another

week of conditioning, as indicated by no difference between groups in the percent time on the cocaine floor at Post-test 2 (enkephalin versus vehicle: $t_{28} = 0.75$, $p = 0.5$; Figure 6C).

In Figure 1, we showed that short-term cocaine exposure followed by withdrawal reduces striatal *Penk* expression. We wondered whether short-term cocaine without withdrawal, like in our CPP experiments, would also lower striatal *Penk* and could potentially be biasing our results. C57Bl/6J mice were treated with cocaine (15 mg/kg) for 5 days, and striatal *Penk* expression was measured immediately after the final treatment. In contrast to the effect of short-term cocaine followed by withdrawal, short-term cocaine in absence of withdrawal did not alter *Penk* expression relative to saline-treated controls ($t_7 = 0.78$, $p = 0.5$; Figure S5). This was not due to poor injections, as we observed a robust cocaine-induced locomotion that sensitized across days compared with saline-treated controls (2W-Mixed Model, day \times drug: $F_{6,197} = 60.01$, $p < 0.0001$; day 1 versus day 5 for cocaine: $t_{17} = 7.95$, $p < 0.0001$; Figure S5). Thus, it is unlikely that the short-term cocaine exposure in our CPP experiments lowered *Penk* expression levels in vehicle-treated controls and accounted for the differences between treatment groups.

DISCUSSION

A large literature implicates low availability of D2Rs in the striatum as a vulnerability factor for developing SUDs; however, the downstream circuit mechanisms driving this vulnerability have remained elusive. Here, we provide evidence that both the levels of *Drd2* mRNA expression and the degree of D2R activation by cocaine in striatal neurons bidirectionally regulate expression of enkephalin within the striatum. Overexpression of D2Rs in striatal neurons led to decreased *Penk* mRNA, while selective loss of D2Rs from D2-MSNs increased *Penk* mRNA. Further, naloxone more potently enhanced striatal GABA transmission and conditioned a place aversion in MSN-*Drd2*KOs relative to controls, indicating that low striatal D2Rs increase the tone of enkephalin in the striatum. The length of cocaine treatment was associated with a similar bidirectional change in striatal *Penk* mRNA and was dependent on the activation of D2Rs expressed in D2-MSNs.

Increasing the enkephalin tone within the slice inhibited GABA transmission between striatal neurons. This may be an important mechanism for promoting cocaine seeking because we previously showed that suppressed collateral GABA transmission decreases lateral inhibition onto D1-MSNs, facilitates striatal output, and promotes cocaine behavioral responses (Dobbs et al., 2016, 2017, 2019). In agreement with this, artificially enhancing striatal enkephalin tone during cocaine place conditioning trials potentiated the acquisition of CPP, while antagonizing striatal μ -opioid receptors blocked expression of CPP in wild-type mice. Together, this suggests enhanced striatal enkephalin tone is a mechanism that contributes to heightened cocaine reward and could promote risk to develop a cocaine SUD.

Previous reports indicate that global D2R knockout mice or treatment with non-selective D2-like antagonists increase *Penk* expression in the striatum (Baik et al., 1995; Romano et al., 1987). Here, we show that selective deletion of D2Rs expressed in D2-MSNs is sufficient to enhance striatal *Penk* expression, while, conversely, overexpression of D2Rs in striatal neurons decreases *Penk*. Interestingly, withdrawal from long-term and short-term

cocaine treatment had a similar bidirectional influence over striatal *Penk* expression. We suspect long-term cocaine increased *Penk* by downregulating striatal D2R availability or decreasing D2R sensitivity, as previously reported (Gong et al., 2021; Moore et al., 1998; Nader and Czoty, 2005). Conversely, withdrawal from short-term cocaine decreases *Penk* expression in the striatum of wild-type mice, but has no effect on *Penk* levels in mice with low striatal D2Rs. These data recapitulate the striatal D2R overexpression findings and further support the mechanism that cocaine is affecting *Penk* expression by altering the activity and expression of D2Rs expressed in D2-MSNs.

Factors determining whether cocaine increases or decreases *Penk* expression likely include duration of exposure, total cocaine received, and withdrawal time. Previous studies found that longer cocaine exposure, either via self-administration (Crespo et al., 2001; Mantsch et al., 2004) or non-contingent injections (Mathieu-Kia and Besson, 1998; Mongi-Bragato et al., 2016), increased *Penk* mRNA in the striatum. We propose that longer cocaine exposure, higher cumulative dose, and/or longer withdrawal would downregulate or desensitize D2Rs selectively in D2-MSNs in response to the chronically heightened dopamine levels in the striatum, and this would in turn increase *Penk* expression. Thus, the activity of striatal D2Rs appears to tightly regulate the function of *Penk* gene. While it is unclear what is driving this interaction, experiments using cell culture suggest that elevated levels of cAMP-dependent protein kinases facilitate *Penk* transcription (Eiden and Hotchkiss, 1983; Eiden et al., 1984; Quach et al., 1984).

This cell-intrinsic adaptation in response to altered dopamine transmission has been previously suggested in reports with dopamine-depleted mice. Enhanced *Penk* expression following 6-OHDA lesions is proposed as an adaptive mechanism to maintain balance in striatal output, as it correlates with partial recovery of motor impairment (Steiner and Gerfen, 1998). However, this *Penk* enhancement may also generate vulnerability to cocaine seeking by suppressing collateral GABA transmission onto D1-MSNs and ultimately promoting their output. This is supported by our electrophysiology data showing that bath application of met-enkephalin or the m-opioid agonist DAMGO, or potentiation of endogenous opioids suppresses GABA transmission onto putative D1-MSNs.

Naloxone more effectively increased spontaneous GABA transients in MSN-*Drd2*KO mice compared with controls, suggesting that pre-existing low levels of striatal D2Rs enhance opioid peptide tone. However, the peptidase inhibitor-mediated suppression of optically evoked GABA was not blocked by naloxone, or receptor antagonists for other common striatal peptides like Substance P and nociceptin. Thus, it appears another peptide is upregulated in MSN-*Drd2*KO mice that is suppressing collateral GABA. One potential candidate is the cocaine-amphetamine regulated transcript (CART) peptide, which is highly expressed in the NAc and associated with blunting the behavioral responses to cocaine (Jaworski et al., 2003; Yoon et al., 2007, 2010).

MSN-*Drd2*KO mice are also more sensitive to naloxone place aversion, indicating that alterations in striatal enkephalin tone may shift an organism's hedonic set-point. This is supported by reports that opioid receptors and peptides localized to the NAc shell and ventral pallidum are important regulators of hedonic motivation (Castro and Berridge, 2014;

Castro and Bruchas, 2019; Peciña and Berridge, 2005). Consistent with this, antagonizing m-opioid receptors in the NAc shell with CTAP blocked expression of cocaine CPP in wild-type mice. In addition, augmenting enkephalin tone selectively within the NAc shell was sufficient to potentiate the acquisition, but not the maintenance, of CPP. It is important to note that the effect size was modest likely due to the relatively low enkephalin dose (5 µg met-enkephalin acetate salt/side) and restricted infusions to the NAc shell. While this dose was based on the effective concentrations in our electrophysiology experiments, single bolus infusions in the NAc are quite different from continuous bath application in the slice. We speculate that a higher enkephalin dose or an infusion that encompassed a larger striatal region might reveal a larger potentiating effect of enkephalin on cocaine reward. Although we cannot rule out the involvement of the dorsal striatum, these findings are supported by previous studies showing that intra-NAc administration of an MOR agonist or antagonist reinstates or blocks cocaine seeking, respectively (Simmons and Self, 2009; Soderman and Unterwald, 2008), and that rats self-administer met-enkephalin directly into the NAc (Goeders et al., 1984). It is also possible we encountered a ceiling effect for cocaine preference; however, in our experience it is difficult to establish dose-responses for cocaine CPP. Regardless, the combination of place preference experiments presented here provide strong convergent evidence for a role of opioid peptides in the striatum, and enkephalin in particular, in supporting the acquisition and maintenance of conditioned cocaine reward.

The ability of enkephalin to enhance acquisition of CPP is similar to the MSN-*Drd2*KO mice phenotype, which shows faster acquisition of cocaine preference (Dobbs et al., 2016). Surprisingly, even though MSN-*Drd2*KO mice have heightened striatal enkephalin and faster acquisition of cocaine CPP, a MOR antagonist in the NAc did not block development of cocaine CPP. It is possible that in the MSN-*Drd2*KO, enkephalin signaling through MORs in the dorsal striatum was sufficient to facilitate cocaine CPP; however, this is unlikely since systemic naloxone failed to block expression of cocaine CPP. We suspect the heightened enkephalin tone in MSN-*Drd2*KO mice may have induced plasticity of DORs, or may be acting in concert with other neuroadaptations, to potentiate cocaine reward. Indeed, repeated cocaine exposure induces a DOR-mediated LTD of GABA transmission from D2-MSNs to the ventral pallidum (Creed et al., 2016). In addition, MSN-*Drd2*KO mice have enhanced D1-mediated signaling (Dobbs et al., 2019), which could mask the effect of opioid receptor antagonists.

Together, these data suggest that low *Drd2* levels in D2-MSNs or long-term cocaine use enhances enkephalin tone in the striatum, which suppresses collateral GABA transmission onto D1-MSNs to facilitate direct pathway output and cocaine reward. This is consistent with enkephalin being necessary for the expression of behavioral and molecular plasticity in striatal neurons following repeated cocaine exposure (Mongi-Bragato et al., 2016). In addition, enkephalins have been shown to affect plasticity of striatal circuits and D1-MSN activity by regulating glutamate transmission via MORs (Atwood et al., 2014; Trieu et al., 2022). Our findings expand on this by demonstrating that enkephalins also regulate collateral GABA transmission onto D1-MSNs. In addition, constitutively active MORs expressed in D2-MSNs due to chronically heightened enkephalin may induce LTD of striatopallidal synapses, as previously seen in rats with a history of cocaine self-administration (Kupchik et al., 2014). Whether a similar MOR-mediated LTD is induced

within striatal GABAergic collaterals is unknown and remains an interesting question for future studies.

We found that withdrawal from short-term cocaine use, which may be akin to a recreational use pattern, attenuates *Penk* levels via activation of D2Rs in D2-MSNs. While it is unclear what effect low striatal enkephalin levels have on the circuit and behavior, it is possible this could suppress cocaine reward and protect against developing problematic cocaine use. However, low striatal enkephalin may drive some to escalate cocaine use in the short term in an attempt to regulate their baseline hedonic set-point. In this case, we predict that those who continue to use cocaine chronically or in high doses would develop downregulated striatal D2Rs, enhanced *Penk* expression, and potentiated cocaine reward. Further, the proclivity toward cocaine use in those with pre-existing low striatal D2R availability might suggest that enkephalin levels are already high and enhancing cocaine's rewarding properties. Moreover, data from other studies suggest that increased striatal enkephalin due to low striatal D2Rs may be a common mechanism underlying vulnerability to a wide range of SUDs. Low D2R availability in the striatum is reported in individuals with an alcohol (Gleich et al., 2020; Heinz et al., 2005; Volkow et al., 1996, 2007), methamphetamine (Lee et al., 2009; Volkow et al., 2001), nicotine (Fehr et al., 2008), and opiate use disorder (Martinez et al., 2012; Wang et al., 1997), and increased striatal *Penk* mRNA or enkephalin peptide levels are reported following use of nicotine (Petruzzello et al., 2013), methamphetamine (Cadet et al., 2016), and alcohol (Chang et al., 2010; Meinhardt et al., 2015; Palm et al., 2012). Thus, D2R-mediated changes in striatal enkephalin and the resulting alterations in collateral GABA transmission may represent a unifying neural circuit mechanism mediating the risk for SUD. These data reveal the complex nature of enkephalin signaling in the striatum and suggest that heightened striatal enkephalin due to low D2Rs or extended cocaine use can have long-term effects on MSN activity, striatal output, and drug seeking and taking.

Limitations of the study

Our electrophysiology experiments used pharmacological approaches to infer heightened enkephalin tone in the striatum. We found that bath application of peptidase inhibitors reduced the frequency of spontaneous GABA events, and this was reversed by naloxone. We interpret these findings as evidence for heightened opioid peptide tone acting on presynaptic opioid receptors in D2-MSNs to restrain GABA release. While we found that MSN-*Drd2*KO mice also have heightened *Penk* gene expression, we cannot rule out a role for other opioids affecting GABA transmission. Opioid peptides responsible could be met- or leu-enkephalin from D2-MSNs, dynorphins or leu-enkephalin from D1-MSNs, and β -endorphin from striatal afferents. In addition, since recordings were performed in putative (ChR2-negative) D1-MSNs, it remains unclear whether enhanced enkephalin tone similarly inhibits collateral GABA onto D2-MSNs. Future work using genetically encoded fluorophores to label D1- and D2-MSNs will address the complexity of enkephalin's actions at specific striatal synapses.

In addition, although MSN-*Drd2*KO mice were more sensitive to develop naloxone place aversion, we cannot rule out a contribution from dynorphin or β -endorphin. However, given these mice have normal *Pdyn* mRNA and robustly enhanced *Penk* mRNA, we suspect

this effect is mediated by heightened enkephalin. We also observed that enhancing the enkephalin levels selectively in the NAc only modestly potentiated cocaine CPP acquisition. This may indicate that enkephalin in the NAc and dorsal striatum work together to support cocaine reward. Future experiments using MSN-selective *Penk* knockout mice will directly address the contribution of striatal enkephalin to cocaine seeking and taking and collateral GABA transmission.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information regarding reagents and resource sharing should be directed to and will be fulfilled by the Lead Contact, Dr. Lauren Dobbs (ldobbs@austin.utexas.edu).

Materials availability—This study did not generate new materials.

Data and code availability—All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals—Adult (8–21 week) mice were used in all experiments. Male and female mice with a homozygous deletion of the D2R from MSNs (MSN-*Drd2*KO) were generated by crossing *Drd2^{loxP/loxP}* and *Adora2aCre* mice, as previously described (Dobbs et al., 2017; Lemos et al., 2016). To overexpress the D2R, transgenic mice expressing the long form of the human *Drd2* gene were generated as previously described (Kellendonk et al., 2006; Mayford et al., 1996). All mouse lines are available commercially and listed in the Key resources table. Mice expressing the *Drd2* transgene under the tetracycline response element tetO were crossed with mice expressing the tetracycline transactivator (tTA) transgene under the CamKII α promoter. This induced overexpression of the *Drd2* transgene in the striatum of double transgenic tTA/tetO-positive mice (D2R-OE), but not in control mice which carry only one, or neither transgene. Transgene expression was switched off following treatment with the tetracycline analogue doxycycline administered in the chow (40 mg/kg Mutual Pharmaceutical). To test the effect of cocaine on *Penk* expression, male C57Bl/6J (Jackson Laboratory; #000664) or MSN-*Drd2*KOs were used. Experiments were performed in accordance with guidelines from the Animal Care and Use Committees at the University of Texas at Austin, the National Institute on Alcohol Abuse and Alcoholism, and the New York State Psychiatric Institute. Mice were group housed, except cannulated mice, and maintained under a 12:12 h light cycle (6:30 ON/18:30 OFF) with food and water *ad libitum*.

METHOD DETAILS

Stereotaxic cannulation and virus injection—Adult male and female *Drd2^{loxP/loxP}* and MSN-*Drd2*KO mice were placed in a stereotaxic frame under isoflurane anesthesia

and a 26-gauge bilateral guide cannula (Plastics One) was implanted above the NAc shell (AP: +1.34, ML: \pm 1.0, DV: -3.5). Two skull screws and dental cement were used to secure the cannula in place. A dummy stylet was placed into the guide and protected with a dust cap when not in use. Mice were given at least one week after surgery before experimental procedures began. For electrophysiology experiments, adult female MSN-*Drd2*KO mice were stereotaxically infused under isoflurane anesthesia with a Cre-dependent adeno-associated virus (AAV) expressing channelrhodopsin-2-EYFP (rAAV5-EF1-DIO-hChR2(H134R)-EYFP; 4.5×10^{12} , UNC; 300nL/side at 100 nL/min) bilaterally into the NAc core (from bregma: AP: + 1.3; ML: \pm 1.0; DV: -4.6).

Jugular catheterization—Male and female C57Bl/6J mice were surgically implanted with indwelling jugular catheters by inserting the catheter into a hole in the vein made using vannas spring scissors. The catheter was held in place with silk suture thread and fed subcutaneously so the inlet port was fixed between the scapulae. Catheters were constructed from micro-renathane tubing (Braintree Scientific) attached to a 26-gauge pedestal inlet port surrounded by polyester mesh (Plastics One). Catheter patency was tested at the beginning of the experiment by infusing Brevital into the catheter (2% solution, 20 μ L). Mice that failed patency were removed from the study.

Drugs—Doxycycline was administered *ad libitum* as a supplemented chow (40 mg/kg, Mutual Pharmaceutical) for 2 weeks to switch the transgene off. Cocaine (15 mg/kg, Sigma) and saline were administered intraperitoneally (i.p.) at 10 mg/kg body weight. Naloxone, met-enkephalin acetate salt, DL-thiorphan, and bestatin HCl were supplied by Millipore Sigma. Gabazine (SR95531) was supplied by Abcam and CTAP and naloxone were from Tocris. Doses for cocaine and naloxone for place conditioning were selected based on previous experiments (Cunningham et al., 1995; Dobbs et al., 2016; Skoubis et al., 2005; Solecki et al., 2009). The *in vivo* met-enkephalin dose was selected based on our *ex vivo* slice physiology experiments. The CTAP dose was selected based off efficacy in blocking cocaine preference and opioid analgesia in mice and rats (Kramer et al., 1989; Soderman and Unterwald, 2008). Drugs were dissolved in aCSF (electrophysiology) or saline (CPP).

Electrophysiology—Due to limited availability of animals during the COVID-19 pandemic, only males were available for the electrophysiology experiments. Sagittal slices (240 μ m) from adult male *Adora2a*Cre or MSN-*Drd2*KO mice were prepared in cold cutting solution (in mM: 225 sucrose, 119 NaCl, 2.5 KCl, 0.1 CaCl₂, 4.9 MgCl₂, 26.2 NaHCO₃, 1 NaH₂PO₄, 1.25 glucose, and 3 kynurenic acid). Artificial cerebrospinal fluid (aCSF) contained (in mM): 124 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 26.2 NaHCO₃, 1 NaH₂PO₄, and 20 glucose. Slices were maintained at 31–33°C for the duration of recordings. Whole-cell voltage clamp recordings were measured from ChR2-negative MSNs in the NAc core. We excluded cells that had a ChR2 current, were spontaneously active, had an $R_i > 500$ M Ω , or a resting membrane potential > -75 mV. Cells were held at -55 mV and GABA-A receptor-mediated synaptic responses were isolated with synaptic blockers (5 μ M NBQX, 10 μ M CPP, and 2 μ M CGP). Recordings were made using glass electrodes (2.5–3.5 M Ω) filled with an internal solution containing (in mM): 60 CsMS, 60 CsCl, 10 HEPES, 0.2 Cs-EGTA, 4 Na-ATP, 0.4 Na-GTP, and 10 phosphocreatine (pH \sim 7.2, \sim 300 mOsm).

Optically-evoked and spontaneously generated inhibitory post-synaptic currents (IPSCs) were measured during bath application of peptidase inhibitors (5 min; 2 μ M thiorphan + 10 μ M bestatin), met-enkephalin + peptidase inhibitors (5 min; 1 μ M), naloxone (10 min; 5 μ M) and gabazine (5 min; 5 μ M). Optically-evoked IPSCs were triggered every 20 s by a single light pulse (473 nm; 0.2–1ms duration) delivered through a fiber optic (200 μ m/0.22 NA, ThorLabs) connected to a laser (25 mW, CrystaLaser). Data were acquired using Multiclamp 700B (Molecular Devices), filtered at 1 kHz, and digitized at 5 kHz. All data were analyzed using pClamp (Clampfit, v. 10.3) and Mini Analysis (Synaptosoft).

Place conditioning—Male and female mice received either a 5-min habituation session (naloxone place aversion experiments) or a 30-min pre-conditioning preference test (cocaine place preference experiments) before undergoing eight days of an unbiased conditioning procedure in a two-chamber apparatus (Med Associates) with distinct tactile floor cues (Grid or Hole). For naloxone place aversion conditioning, mice received saline (CS-) or naloxone (CS+, 1 mg/kg) and were confined to either the Grid or Hole floor for 30 min. For cocaine place conditioning, mice received saline (CS-) or cocaine (CS+, 15 mg/kg) and were confined to the Grid or Hole floor for 15 min. Floor orientation (Grid on left or right), drug order (cocaine on first or second day), and drug-cue pairing (cocaine with Grid or Hole) were counterbalanced across mice. Saline-primed preference tests were administered after 4 and/or 8 days of conditioning, wherein mice have access to both floor types for 30 min. Additional drug-primed tests were also performed on the last experiment day in the naloxone place aversion experiment and the CTAP experiments. Preference is calculated as the percent time on the cocaine-paired floor, and also as the time spent on the grid floor for each conditioning subgroup (Grid + Drug or Hole + Drug). A significant difference in the time spent on the grid floor between these two conditioning groups indicates place conditioning (Cunningham et al., 2003). For example, if mice that had cocaine paired with the grid floor spend significantly more time on the grid floor than mice that had cocaine paired with the hole floor, this would be indicative of place preference.

To determine the role of enkephalin and MORs in the acquisition and expression of cocaine CPP, we infused the MOR antagonist CTAP or a met-enkephalin/peptidase inhibitor cocktail into the NAc shell during cocaine conditioning trials or preference tests in two different experiments. In the first experiment, *Drd2^{loxP/loxP}* mice were conditioned with cocaine over 8 days. Mice then received a preference test, which involved an intra-NAc vehicle infusion immediately followed by saline (i.p.). The next day, mice were tested again after receiving an intra-NAc CTAP infusion immediately followed by saline (i.p.). In the second experiment, *MSN-Drd2KO* mice were conditioned with cocaine over 8 days. On CS+ days, each mouse received cocaine (15 mg/kg, i.p.) immediately after an intra-NAc infusion of either saline (NAc Veh + Coc group) or CTAP (NAc CTAP + Coc group). On CS- days, all mice received saline (10 mL/kg, i.p.) immediately following a sham microinjection into the NAc, wherein a shortened bilateral injector was inserted into the cannula that did not extend into the tissue. Saline-primed preference tests were administered after 4 (Test 1) and 8 (Test 2) conditioning days, and a naloxone-primed test was given the last experimental day. Mice received sham intra-NAc injections followed by i.p. injections on preference test days. In the third experiment, *Drd2^{loxP/loxP}* mice were conditioned with cocaine over 8 days. On CS+

days, cocaine was given immediately after intra-NAc infusion of met-enkephalin cocktail (NAc Enk + Coc group). CS- days and saline-primed preference test were administered as in Experiment 2.

Intra-NAc microinjection procedure: Intra-NAc vehicle, enkephalin or CTAP was administered by gently restraining mice and inserting a bilateral microinjector into the NAc shell that extended 1.5 mm beyond the end of the cannula. Saline, CTAP (300 ng/side), or a met-enkephalin cocktail (5 µg met-enkephalin acetate salt + the peptidase inhibitors: 150 ng DL-thiorphan, 1 µg bestatin HCl) was infused over 3 min (100 nL/min) via a dual syringe pump. Peptidase inhibitors were included with met-enkephalin to prevent premature degradation of met-enkephalin. Microinjectors were left in place 2 min after infusion to allow for drug diffusion. Stylets and dust caps were replaced and then mice were administered either saline or cocaine and were placed into the preference chamber.

Contingent and non-contingent cocaine treatment—To determine the effect of withdrawal from non-contingent long-term and short-term cocaine exposure on striatal *Penk* expression, C57Bl/6J mice and MSN-*Drd2*KO mice were administered saline or cocaine injections for either 5 days (15 mg/kg, i.p.) or 10 days (20 mg/kg, i.p.). For these experiments, injections occurred in the animal's light cycle and mice were immediately returned to their home cage after injection. To determine whether contingently administered cocaine affects striatal *Penk* expression, mice were trained to self-administer cocaine intravenously in an operant chamber equipped with two retractable levers and cue lights positioned over each lever (Med Associates). Mice were allowed to lever press for cocaine under a fixed ratio 1 schedule of reinforcement, wherein a single press of the active lever resulted in illumination of the cue light and delivery of a cocaine infusion (1 mg/kg/infusion). A separate group of mice were yoked saline controls, which received saline infusions in the same pattern as a yoked cocaine-administering animal. Self-administration occurred in the animal's dark cycle over 10 days in daily 2-h sessions. For these contingent and non-contingent experiments, brains were extracted for qPCR analysis 14 days after their last injection. To determine how short-term cocaine in absence of withdrawal affects striatal *Penk* expression, mice first had 3 days of saline injections (i.p., 10mL/kg) followed by 5 days of saline or cocaine (15 mg/kg, i.p.). Locomotor activity was measured in an open field apparatus (28 cm × 28 cm; Med Associates) for 1 h after each injection in the animal's light cycle. Brains were extracted for qPCR analysis immediately after the last injection.

Quantitative polymerase chain reaction—Mice were euthanized with isoflurane, decapitated and brains were extracted. The striatum was dissected on ice and stored in RNA-later Stabilization Solution (ThermoFisher) for 24 h at 4°C, then at -80°C until extraction. RNA was purified using a RNeasy Plus Mini kit (Qiagen) and cDNA was synthesized using iScript Reverse Transcription Supermix (Biorad) or the Invitrogen Superscript II First Strand Synthesis System (Thermo Fisher). For real time PCR of samples from MSN-*Drd2*KO or C57BL/6J mice, TaqMan Gene Expression Assays (Thermo Fisher) or PrimePCR Probe Assays (Bio-Rad) were used to quantify relative gene expression using a StepOnePlus Real-Time PCR system (Applied Biosystems) or a CFX384 (Bio-Rad). The specific probes used were: from Thermo Fisher, beta

actin (Mm01205647), preproenkephalin (Mm01212875_m1), dopamine D2 receptor (Mm00438541_m1), delta opioid receptor (Mm00443063_m1), and mu opioid receptor (Mm01188089_m1), prodynorphin (Mm00457573_m1), and from Bio-Rad, dopamine D2 receptor (qMmuCIP0030279). For the D2R-OE experiment, SYBR Green PCR Master Mix (Applied Biosystems) was used to quantify *Penk* expression and real time PCR was carried out on a DNA engine with chromo4 real time detection system (Bio-Rad). Primer sequences used included: PPE1: AAGCCCTTTTCCAGCAGTGA, PPE2: TGTACAGCACAAAGCAGCATGT, GAPDH1: TGCAGTGGCAAAGTGGAGATT, GAPDG2: TTGAATTTGCCGTGAGTGGA. All real time PCR reactions were run in triplicate with GAPDH or beta actin run in parallel as a reference. Relative expression of each gene of interest was calculated using the 2^{-Ct} method (Livak and Schmittgen, 2001).

Gene chip analysis—Total RNA from each of 10 mice (5 control, 5 D2R-OE) was amplified and transcribed into cRNA using the MessageAmp II Biotin Enhanced Kit (Ambion) and hybridized with 10 Affymetrix 430A2.0 microarrays in the Gene Chip Facility of the Columbia Genome Center. The raw data was then analyzed using dChip (<https://sites.google.com/site/dchipsoft/>) and ErmineG (<https://erminej.msl.ubc.ca/>).

QUANTIFICATION AND STATISTICAL ANALYSIS

Analyses were performed using Prism (v.8, GraphPad). In consideration of sex as a biological variable (SABV), power and effect sizes were calculated from datasets that included both sexes to determine whether the datasets were sufficiently powered (> 0.8) to detect differences between sex. While males and females were used in most experiments, none of these datasets were sufficiently powered to detect sex differences, and thus data were not analyzed with sex as a statistical factor (Table S1).

For data that met assumptions for parametric tests, one-way, two-way, or three-way analysis of variance (ANOVA) was used, with repeated measures when appropriate, to analyze data with more than 2 groups or factors. When repeated measures data had missing values, a mixed-effects model was used. Significant main effects and interactions were followed-up with pairwise t-tests corrected for multiple comparisons (Sidak). Violations to sphericity in 3-way repeated measures ANOVAs (or mixed models) were corrected using Greenhouse-Geisser. Paired, unpaired, or one-sample t-tests, were used to analyze pairwise data, two independent samples, and comparisons against a single value, respectively. Corrections for unequal variance were made using Welch's correction when appropriate. For data that did not meet assumptions for parametric tests (*Adora2a-Cre* sIPSC distributions in Figures 2 and 3), non-parametric alternatives were used. Wilcoxon signed rank test was used to compare sIPSCs before and after drug application, and the Kolmogorov-Smirnov test was used to compare distributions between genotypes. Data are presented as the mean \pm SEM, and results were considered significant at an alpha of 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Dopamine D2Rs bidirectionally regulate striatal enkephalin expression
- Endogenous enkephalin suppresses intra-striatal GABA transmission
- Heightened striatal enkephalin levels potentiate cocaine reward in wild-type mice
- Opioid receptor antagonists attenuate cocaine reward in wild-type mice

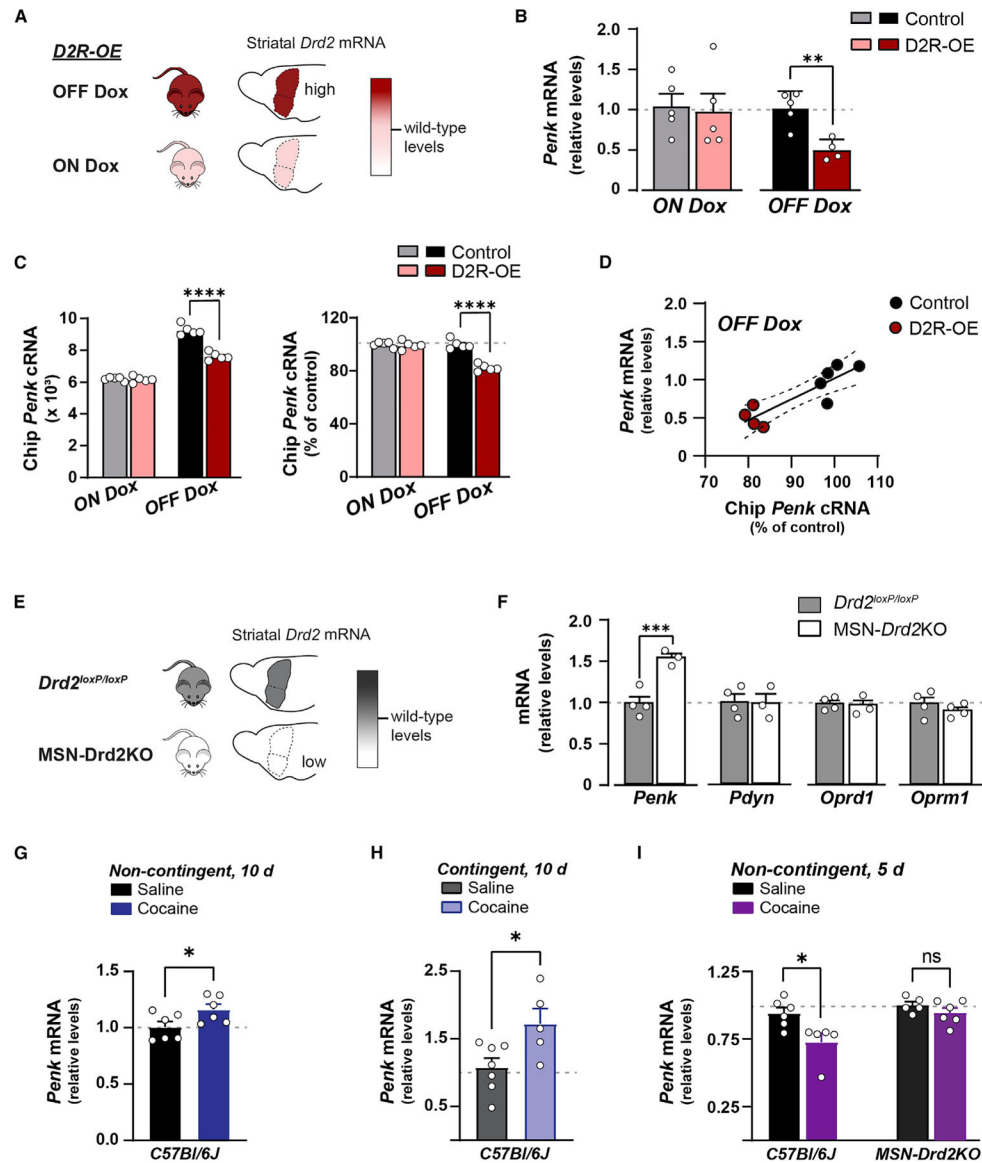


Figure 1. Dopamine D2 receptor activity in medium spiny neurons bidirectionally regulates striatal preproenkephalin expression

Expression of specific genes is quantified with qPCR and gene chip microarray for mice overexpressing D2Rs (A–D) or following selective loss of D2Rs from D2-MSNs (E and F).

(A) Schematic shows *Drd2* mRNA levels are increased over wild type (pink) in mice expressing the human *Drd2* transgene (D2R-OE) and in the absence of doxycycline-supplemented chow (dark red).

(B) Striatal preproenkephalin (*Penk*) mRNA levels in D2R-OE mice (n = 4–5) or controls (n = 5). When *Drd2* transgene expression was suppressed (ON Dox, pink) *Penk* mRNA was similar to controls. Turning on *Drd2* transgene (OFF Dox, dark red) reduced *Penk* mRNA (B) and cRNA (C) compared with controls (t test).

(D) Correlation of *Penk* levels between gene chip microarray and qPCR when the transgene is expressed (OFF Dox, red) in D2R-OE and control mice.

(E) Schematic shows decreased *Drd2* mRNA levels in mice with a homozygous deletion of *Drd2* from D2-MSNs (MSN-*Drd2*KO, white) relative to wild type (gray).

(F) MSN-*Drd2*KO mice (n = 3) show increased *Penk* mRNA, but no change in dynorphin (*Pdyn*) or δ - and μ -opioid receptors (*Oprd1*, *Oprm1*) compared with littermate controls (gray, n = 4; t test).

(G–I) Ten days of non-contingent (G; 20 mg/kg, intraperitoneally) or contingent (H; mean: 17 ± 3 mg/kg, intravenous self-administration) cocaine followed by 14 days of withdrawal increased striatal *Penk* mRNA in C57Bl/6J. (I) Five days of non-contingent (15 mg/kg, intraperitoneally) cocaine followed by 14 days of withdrawal reduced *Penk* mRNA in C57Bl/6J, but not MSN-*Drd2*KO mice (J). ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Mean \pm SEM and/or individual values shown.

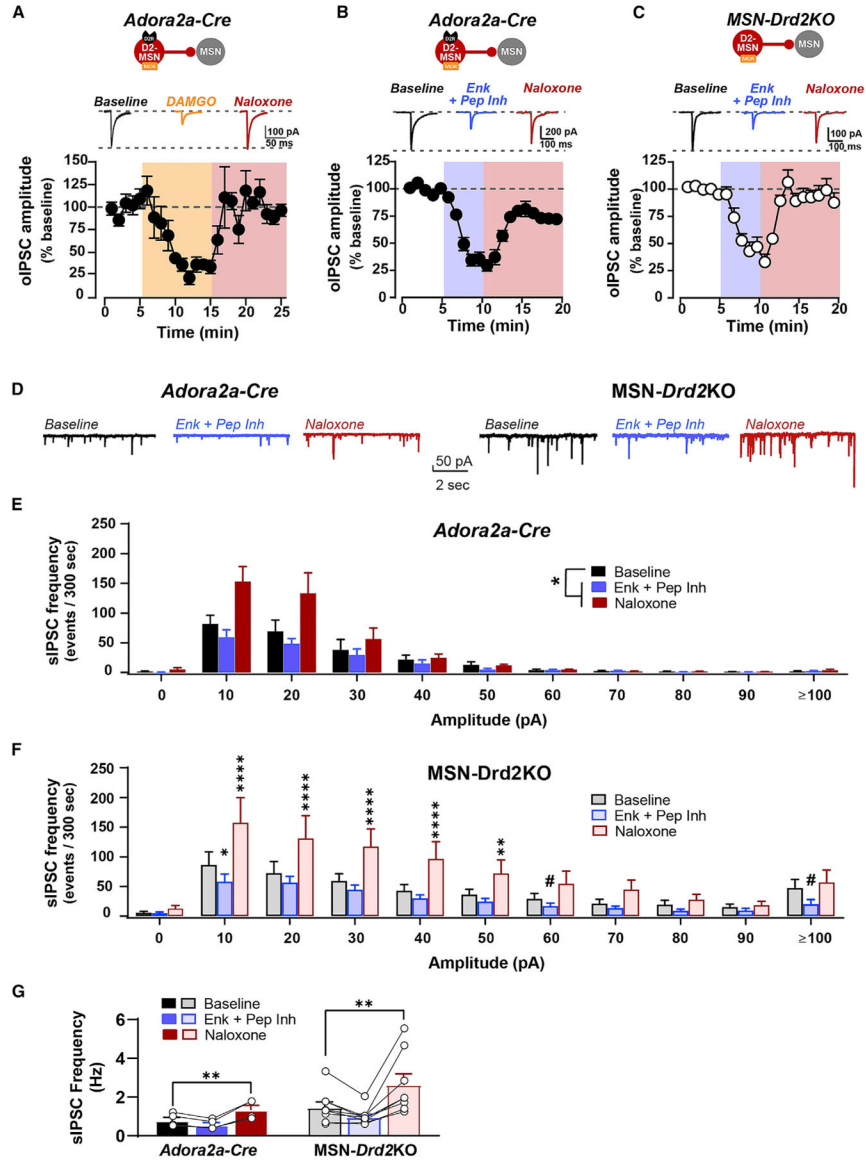


Figure 2. Enkephalin and MOR agonist suppress intra-striatal GABA transmission
 (A–C) Top, Representative oIPSC traces recorded from MSNs in *Adora2a-Cre* or *MSN-Drd2KO* in presence of the μ -opioid receptor agonist DAMGO (1 μ M, orange), enkephalin (1 μ M) + peptidase inhibitors (2 μ M thiorphan + 10 μ M bestatin; blue), or the opioid receptor antagonist naloxone (5 μ M; red). Bottom, Time course of oIPSC amplitude expressed as percent of baseline recorded from ChR2-negative MSNs from *Adora2a-Cre* (n = 5 cells/2 mice) and *MSN-Drd2KO* mice (C, n = 10 cells/3 mice).
 (D) Representative spontaneous IPSC (sIPSC) traces recorded from MSNs in *Adora2a-Cre* (left) or *MSN-Drd2KO* (right) are shown in the presence of enkephalin + peptidase inhibitors (blue) or naloxone (red).
 (E and F) Number of sIPSCs plotted by amplitude for *Adora2a-Cre* (left, n = 4 cells/2 mice) or *MSN-Drd2KO* (right, n = 8 cells/3 mice) during enkephalin + peptidase inhibitors (E) or naloxone (F) relative to peptidase inhibitor baseline (black).

(G) sIPSC frequency was enhanced by naloxone in both genotypes. Parametric and non-parametric tests performed (see text). #p = 0.05–0.07, *p < 0.05, **p < 0.01, ****p < 0.0001. Mean ± SEM and/or individual values/traces shown.

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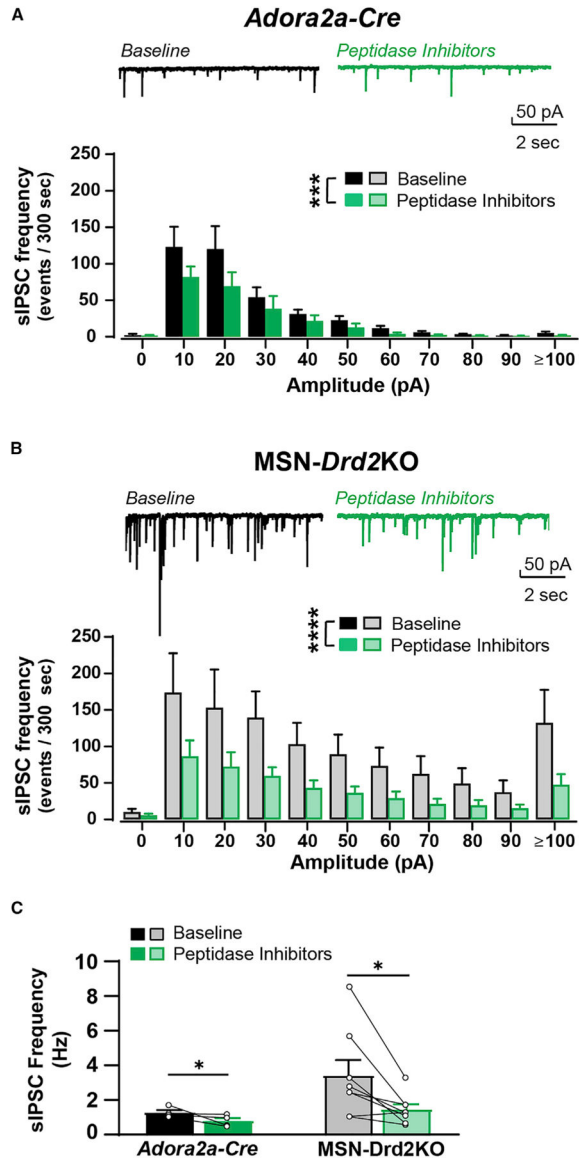


Figure 3. Endogenous peptides suppress spontaneously evoked GABA in the striatum (A and B) Top, Representative spontaneous IPSC (sIPSC) traces recorded from MSNs in *Adora2a-Cre* (A) or *MSN-Drd2KO* (B) in presence of peptidase inhibitors (2 μ M thiorphan, 10 μ M bestatin; green). Bottom, sIPSCs plotted by amplitude for *Adora2a-Cre* (A, n = 4 cells/2 mice) or *MSN-Drd2KO* (B, n = 8 cells/3 mice) at baseline (black) and during peptidase inhibitors. (C) sIPSC frequency is inhibited by peptidase inhibitors for both genotypes. Parametric and non-parametric tests performed (see text) $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Mean \pm SEM and/or individual values/traces shown. See also Figure S1.

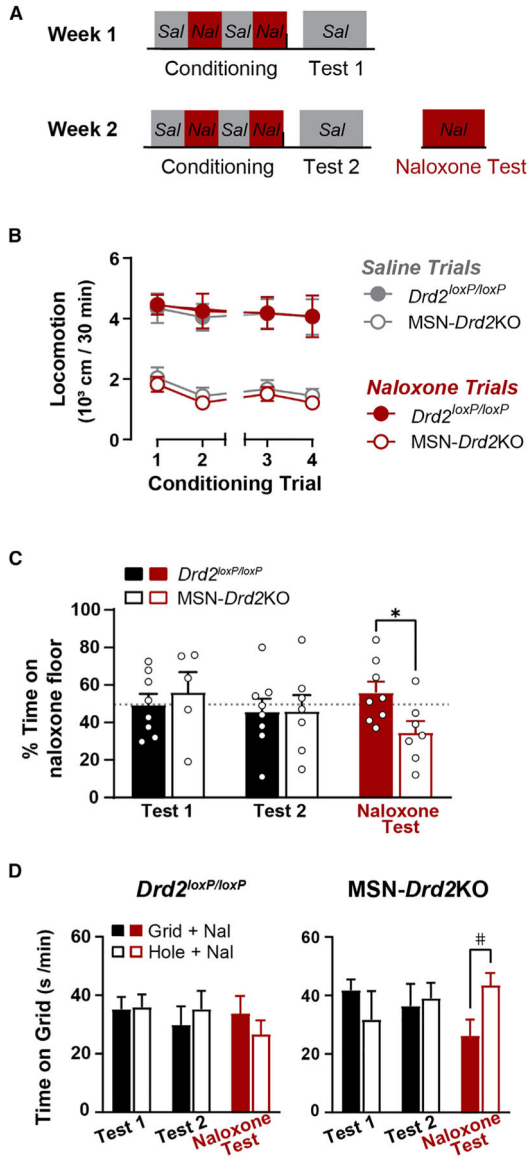


Figure 4. MSN-*Drd2KO* mice are more sensitive to aversive properties of naloxone

(A) Timeline of behavioral experiment.

(B) Locomotor activity during saline (gray) and naloxone (red) conditioning trials is shown for *Drd2^{loxP/loxP}* (filled) and MSN-*Drd2KO*s (open) (3W-RMANOVA).

(C) Percent time on the naloxone paired floor for *Drd2^{loxP/loxP}* (filled, n = 8) and MSN-*Drd2KO* (open, n = 5–7) at Test 1, Test 2, and naloxone test (red) (t tests).

(D) Time on grid floor is shown across preference tests for *Drd2^{loxP/loxP}* (left) and MSN-*Drd2KO* (right) that had naloxone paired with grid floor (filled, Grid + Nal, n = 3–4) or hole floor (open, Hole + Nal, n = 3–4) (2W-ANOVAs). #p = 0.08, *p < 0.05. Mean ± SEM and/or individual values/traces shown. See also Figure S2.

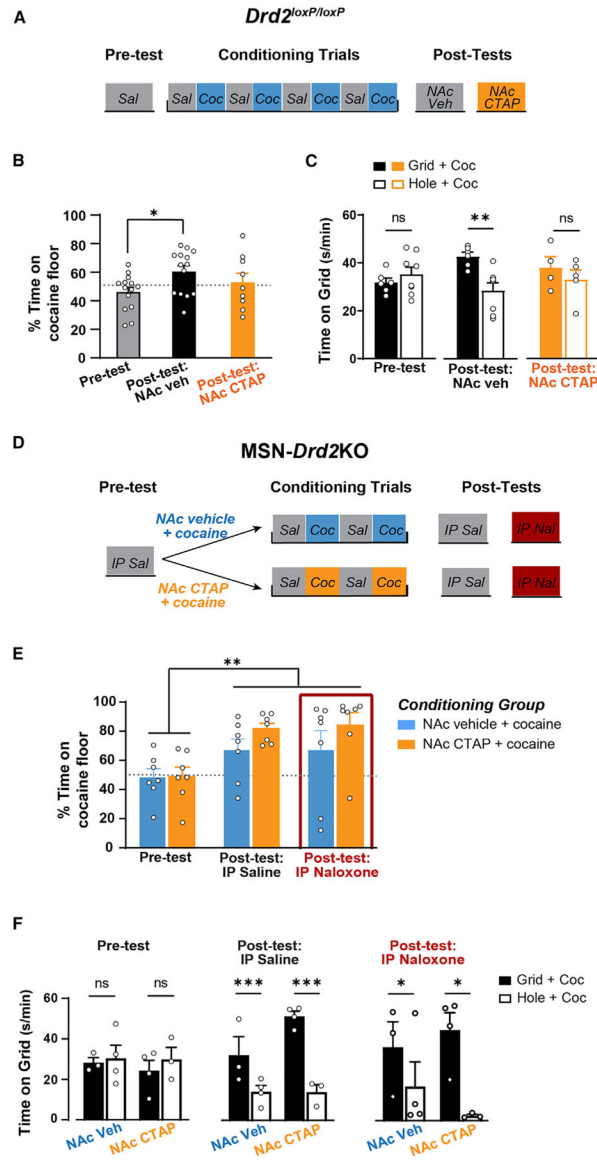


Figure 5. Mu-opioid receptor antagonist in the nucleus accumbens blocks cocaine place preference in wild-type, but not MSN-Drd2Kos

(A) Timeline of behavioral experiment in *Drd2^{loxP/loxP}* mice.

(B) Percent time on cocaine (15 mg/kg) paired floor is shown for Pre-test (gray, n = 14), Post-test (black, n = 14), and CTAP test (300 ng/side; orange, n = 9) (RMANOVA).

(C) Time on grid floor is shown for mice that had cocaine paired with grid floor (filled, Grid + Cocaine, n = 4–6) or with hole floor (open, Hole + Cocaine, n = 5–8) during Pre-test, Post-test, and CTAP test (2W-ANOVAs).

(D) Timeline of behavioral experiment in *MSN-Drd2KO* mice.

(E) Percent time on cocaine (15 mg/kg) paired floor is shown for Pre-test (n = 7/group), Post-test (n = 7/group), and naloxone test (10 mg/kg; red box, n = 7/group) (2W-RMANOVA). Mice had cocaine paired with either intra-NAc vehicle (blue, n = 7) or intra-NAc CTAP (300 ng/side; orange, n = 7).

(F) Time on grid floor is shown for mice that had cocaine paired with grid floor (filled, Grid + Cocaine, $n = 3-4/\text{group}$) or with hole floor (open, Hole + Cocaine, $n = 3-4/\text{group}$) during Pre-test, Post-test, and naloxone test (2W-ANOVAs). Ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Mean \pm SEM and/or individual values shown. See also Figure S3.

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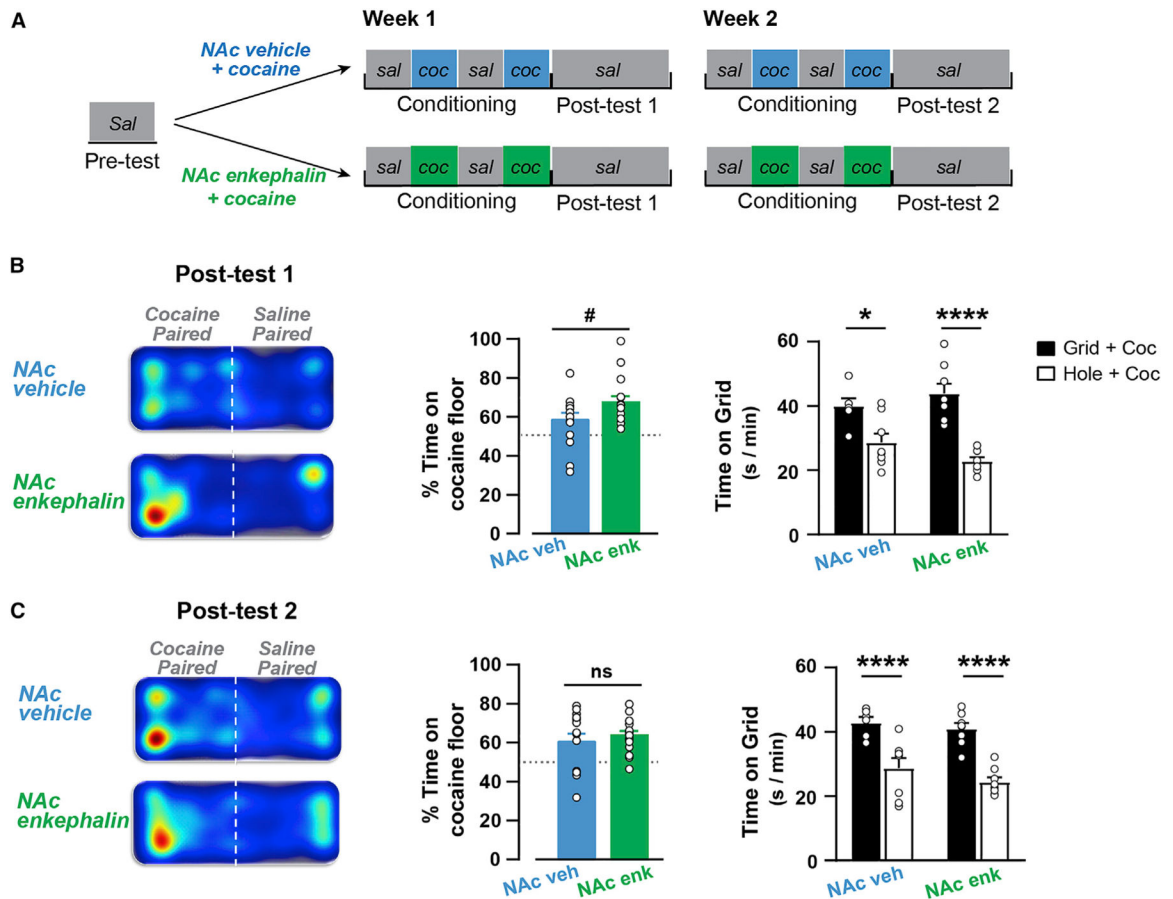


Figure 6. Enkephalin in the nucleus accumbens facilitates acquisition of cocaine place preference (A) Timeline of behavioral experiment in *Drd2^{loxP/loxP}* mice.

(B and C) Left, Representative heatmaps of animal position on cocaine- or saline-paired floors of the conditioning chamber are shown for NAc vehicle group and NAc enkephalin group during Post-test 1 (B) and Post-test 2 (C). Middle, Percent time on cocaine-paired floor for NAc vehicle group (blue, n = 9–14) and NAc enkephalin group (orange, n = 11–16) at Post-test 1 (B) and Post-test 2 (C) (t tests). Right, Time on grid floor is shown for mice that had cocaine paired with grid floor (filled, Grid + Cocaine, n = 6–8) or with hole floor (open, Hole + Cocaine, n = 8) for NAc vehicle group and NAc enkephalin group (2W-ANOVA). ns = not significant, #p = 0.06, *p < 0.05, ****p < 0.0001. Mean ± SEM and/or individual values shown. See also Figures S4 and S5.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
rAAV5-EF1-DIO-hChr2(H134R)-EYFP	UNC Vector Core	n/a; https://www.med.unc.edu/genetherapy/vectorcore/in-stockaaav-vectors/deisseroth/
Chemicals, peptides, and recombinant proteins		
Cocaine HCl	Millipore Sigma	Cat #: C5776
Saline	Hospira	NDC 0409-4888-06
iScript Reverse Transcription Supermix	Biorad	Cat #: 1708840
Met-enkephalin acetate salt hydrate	Millipore Sigma	Cat #: M6638
DL-thiorphan	Millipore Sigma	Cat #: T6031
Bestatin HCl	Millipore Sigma	Cat #: B8385
Naloxone HCl	Tocris	Cat #: 0599
CTAP	Tocris	Cat #: 1560
Gabazine (SR95531)	Abcam	Cat #: ab120042
NBQX disodium salt	Abcam	Cat #: ab120046
(R)-CPP	Tocris	Cat #: 0247
CGP 55845 HCl	Abcam	Cat #: ab120337
Aprepitant	Tocris	Cat #: 6486
J 113397	Tocris	Cat #: 2598
RNAlaterStabilization Solution	Ambion	Cat #: AM7020
TaqMan Gene Expression Assays: preproenkephalin	Thermo Fisher	Mm01212875_m1
TaqMan Gene Expression Assays: beta actin	Thermo Fisher	Mm01205647
TaqMan Gene Expression Assays: dopamine D2 receptor	Thermo Fisher	Mm00438541_m1
TaqMan Gene Expression Assays: delta 1 opioid receptor	Thermo Fisher	Mm00443063_m1
TaqMan Gene Expression Assays: mu 1 opioid receptor	Thermo Fisher	Mm01188089_m1
TaqMan Gene Expression Assays: prodynorphin	Thermo Fisher	Mm00457573_m1
PrimePCR Probe Assay: Drd2, mouse	Bio Rad	qMmuCIP0030279
SYBR Green Master Mix	Applied Biosystems	Fisher cat# 4309155
Invitrogen Superscript II First Strand Synthesis System	Thermo Fisher	11904018
DNase I	Thermo Fisher	89836
Critical commercial assays		
RNeasy Clean-Up Kit	QIAGEN	74204
RNeasy Plus Mini Kit	QIAGEN	Cat #: 74136
MessageAmp II Biotin Enhanced Kit	Ambion	AM1791
Affymetrix 430A2.0 microarrays	Affymetrix (acquired by Thermo Fisher)	Not available
Experimental models: Organisms/strains		
Mouse: Adora2a-Cre; Tg(Adora2a-cre)KG139Gsat/Mmucd	MMRRC	Cat# 031168-UCD; RRID:MMRRC_031168-UCD

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: Drd2loxP/loxP; B6.129S4(FVB)-Drd2tm1.1Mrub/J	JAX	Cat# 020631; RRID:IMSR_JAX:020631
Mouse: C57Bl/6J	JAX	Cat# 000664; RRID:IMSR_JAX:000664
Mouse: B6;CBA-Tg(Camk2a-tTA)1Mmay/J	JAX	Cat# 003010; RRID:IMSR_JAX:003010
Mouse: B6.Cg-Tg(tetO-DRD2)2-5Kndl/J	JAX	Cat# 028294; RRID:IMSR_JAX:028294
Oligonucleotides		
PPE1 Primer: AAGCCCTTTTCCAGCAGTGA	Sigma Custom Oligo	
PPE2 Primer: TGTACAGCACAAAGCAGCATGT	Sigma Custom Oligo	
GAPDH1 Primer: TGCAGTGGCAAAGTGGAGATT	Sigma Custom Oligo	
GAPDH2 Primer: TTGAATTTGCCGTGAGTGGA	Sigma Custom Oligo	
Software and algorithms		
DNA-Chip Analyzer (dChip) software package	https://sites.google.com/site/dchipsoft/	
ErmineG Gene expression profiling software	https://erminej.msl.ubc.ca/	